Inhibition of DNMT3B and PI3K/AKT/mTOR and ERK Pathways as a Novel Mechanism of Volasertib on Hypomethylating Agent-Resistant Cells

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Abstract

Resistance to hypomethylating agents (HMAs) in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) is a concerning problem. Polo-like kinase 1 (PLK1) is a key cell cycle modulator and is known to be associated with an activation of the PI3K pathway, which is related to the stabilization of DNA methyltransferase 1 (DNMT1), a target of HMAs. We investigated the effects of volasertib on HMA-resistant cell lines (MOLM/AZA-1 and MOLM/DEC-5) derived from MOLM-13, and bone marrow (BM) samples obtained from patients with MDS (BM blasts >5%) or AML evolved from MDS (MDS/AML). Volasertib effectively inhibited the proliferation of HMA-resistant cells with suppression of DNMTs and PI3K/AKT/mTOR and ERK pathways. Volasertib also showed significant inhibitory effects against primary BM cells from patients with MDS or MDS/AML, and the effects of volasertib inversely correlated with DNMT3B expression. The DNMT3B-overexpressed AML cells showed primary resistance to volasertib treatment. Our data suggest that volasertib has a potential role in overcoming HMA resistance in patients with MDS and MDS/AML by suppressing the expression of DNMT3 enzymes and PI3K/AKT/mTOR and ERK pathways. We also found that DNMT3B overexpression might be associated with resistance to volasertib.

Key Words: Volasertib, Hypomethylating agent-resistance, DNA methyltransferase, PI3K/AKT/mTOR, ERK pathway

INTRODUCTION

Two hypomethylating agents (HMAs), azacitidine and decitabine, are currently approved for the treatment of patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). These agents have been considered to be a standard of care in elderly or unfit AML or higher-risk MDS (Estey, 2013). However, the overall response rates of azacitidine or decitabine are reported to be around 50% in most studies, and even the patients with initial responses mostly lose their responses between 9 to 15 months of treatment which leads to dismal prognosis (Kadia et al., 2011; Prébet et al., 2012; Lee et al., 2015). The precise mechanism of inherent or acquired resistance to HMA is not well understood (Qin et al., 2009; Sripayap et al., 2014). Polo-like kinases (PLKs) represent the family of serine-threonine kinases modulating cell cycle progression, mitosis, and cellular response to DNA damage. Of the PLK family, PLK1 is the most extensively characterized kinase which mediates mitotic activity, DNA replication, and chromosome/microtubule dynamics. Increased PLK1 expression was observed in primary solid cancer cells as well as in AML cells, and has been clinically correlated with increased risk of metastases and poor prognosis (Renner et al., 2009; Gjertsen and Schöffski, 2015). In vitro inhibition of PLK1 by pharmacologic inhibitor or small interfering RNA blocked proliferation and induced apoptosis of various cancer cell lines, and reduced the clonogenic potential of primary cells from AML patients (Liu and Erikson, 2003; Renner et al., 2009). Volasertib is a small molecule that competitively inhibits PLK1 and, to a lesser extent, closely related kinases PLK2 and PLK3, and induces cell cycle arrest at the G2/M phase (Van den Bossche et al., 2016). In preclinical in vitro and in vivo studies, volasertib shows significant inhibitory effects against primary BM cells from patients with MDS or MDS/AML, and the effects of volasertib inversely correlated with DNMT3B expression. The DNMT3B-overexpressed AML cells showed primary resistance to volasertib treatment. Our data suggest that volasertib has a potential role in overcoming HMA resistance in patients with MDS and MDS/AML by suppressing the expression of DNMT3 enzymes and PI3K/AKT/mTOR and ERK pathways. We also found that DNMT3B overexpression might be associated with resistance to volasertib.
vivo models of multiple cancers including AML, volasertib has shown promising antitumor activities (Rudolph et al., 2009; Gjertsen and Schöffski, 2015). Clinical trials of volasertib alone or in combination with other agents have been performed (Van den Bossche et al., 2016; Goroshchuk et al., 2019). A phase III study evaluated the effectiveness of volasertib combined with low-dose cytarabine in patients with AML ineligible for intensive chemotherapy. The phase II results showed that combined treatment of volasertib and low-dose cytarabine led to higher response rate and survival than low-dose cytarabine alone (Döhner et al., 2014). The phase III trial also demonstrated higher response rate with volasertib although survival benefit was not proven mainly due to increased early mortality (Döhner et al., 2021).

We previously established HMA-resistant cell lines (MOLM/ AZA-1 and MOLM/DEC-5) from parental MOLM-13 which was derived from the peripheral blood of patients with secondary AML evolved from MDS (MDS/AML) (Hur et al., 2017). The HMAs induce depletion of DNA methyltransferase (DNMT) enzymes, especially DNMT1, after incorporation into DNA or RNA leading to global DNA hypomethylation. There are several types of DNMTs in mammals including DNMT1, DNMT3A, and DNMT3B, which have similar amino acid sequences with diverse regulatory domain on N-terminus. Of the DNMT families, DNMT1 mainly plays a role in maintaining methylation status, while DNMT3A and DNMT3B contribute to de novo methylation (Subramaniam et al., 2014). In our HMA-resistant cell lines, DNMT3B was overexpressed compared to the parent cells which was also observed in several human cancer cells, suggesting a potential relationship between DNMT3B and tumor development. The development of resistance to anti-cancer drugs is an important issue, and unraveling and overcoming the resistance mechanisms is necessary to improve clinical outcomes. In vitro studies indicated that volasertib might be useful in tumors resistant to anti-cancer therapies (Rudolph et al., 2009; Bhola et al., 2015). Several studies have suggested that PLK1 expression is associated with the phosphatidylinositol 3-kinase (PI3K) pathway (Kasahara et al., 2014; Li et al., 2014; Wu et al., 2019), and the PI3K pathway is known to stabilize the DNA methyltransferase (DNMT) 1 protein, a target of HMAs (Sun et al., 2007; Estève et al., 2011). In the present study, we explored the potential efficacy of volasertib in our HMA-resistant cell lines and primary cells from MDS or MDS/AML patients.

MATERIALS AND METHODS

Cell lines and patient BM samples

MOLM-13 (DSMZ, Braunschweig, Germany), MOLM/AZA-1, MOLM/DEC-5, THP-1 (ATCC, Manassas, VA, USA) cell lines were cultured at 37°C in 5% CO2 in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% (v/v) fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific). 293FT cell line (Thermo Fisher Scientific) was cultured at 37°C in 5% CO2 in dulbecco modified eagle medium (Thermo Fisher Scientific) containing 10% (v/v) fetal bovine serum (HyClone) and 1% penicillin/streptomycin (Thermo Fisher Scientific).

Bone marrow (BM) mononuclear cells were isolated from 42 BM samples of MDS patients with ≥5% BM blasts or patients with AML evolved from MDS (Supplementary Table 1). Informed consents were obtained from all patients in accordance with Helsinki Declaration, and the study protocol was approved by Institutional Review Board of Asan Medical Center (2012-0217). The bio specimen and data used in this study was provided by Asan Bio-Resource Center, Korea Biobank Network (Seoul, Korea) (BRC#: 2017-05(143)).

Drugs & cell proliferation assay

Volasertib (BI 6727) was kindly provided from Boehringer Ingelheim (Ingelheim, Ingelheim am Rhein, Germany). Azacitidine (cat. no. A2385), decitabine (cat. no. A3656), and cytarabine (cat. no. SC1768) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell viability was assessed by the luminescent-based CellTiter-Glo system (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Briefly, cells were plated at 1,000-3,000 per well in a 96-well opaque plate and were incubated in complete growth medium. Cells were treated with various concentrations of volasertib, azacitidine and decitabine, and were prepared as a 50 mmol/L stock solution in DMSO. After 72 h, cell viability was determined by measuring luminescent signals with a VICTOR™ X Light (PerkinElmer, Waltham, MA, USA). To evaluate the synergistic effects of each drug combination, the combination indices were calculated by a median dose effect analyses using CalcuSyn (Biosoft, Ferguson, MO, USA). Combination index value of less than 1.0 indicate a synergistic interaction of the combination of two drugs.

Cell cycle analysis

MOLM-13, MOLM/AZA-1, and MOLM/DEC-5 (1×10⁶ cells each) were treated with volasertib for 24 h, fixed with 70% (v/v) ethanol, and stained with 60 μg/mL propidium iodide (Sigma-Aldrich) containing 10 units/mL RNaseA (Promega) for 30 min. The cellular DNA contents of each cell cycle phase were analyzed using a flow cytometer (Becton Dickinson, San Jose, CA, USA) and the distribution of cells in each phase was calculated form DNA content histograms.

Immunoblotting analysis

Cells were lysed with lysis buffer (Cell signaling technology, Danvers, MA, USA). Then, protein samples (20 μg) were separated by SDS-PAGE, and were blotted onto polyvinylidene difluoride membranes (Bio-RAD Laboratories, Hercules, CA, USA). After blocking with 1% (w/v) non-fat dry milk powder for 1 h, the membranes were incubated with primary antibodies overnight at 4°C, then with secondary antibody conjugated with horseradish peroxidase (Enzo Life Sciences, Inc., Farmingdale, NY, USA). Specific antigen-antibody complexes were detected by enhanced chemiluminescence using BioFoX® Chemiluminescent Sensitive Plus HRP (SurModics. IVD. Inc., Eden Prairie, MN, USA). The specific band was visualized using Ez-Capture ST chemiluminescence imaging system (ATTO, Tokyo, Japan). Specific antibodies are presented in Supplementary Table 1. Experiments were performed in repeated three times with similar results.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

All the RNA samples were extracted using RNeasy® Plus Mini Kit (Qiagen Gmbh, Hilden, Germany) and converted into cDNA using RevertAid First Strand cDNA Synthesis Kit.
DNMT3A and DNMT3B transduction
To clone the genes encoding DNMT3A and DNMT3B in lentivirus, the plasmids were designed and synthesized by cloning service (Cosmogenetech Inc., Seoul, Korea). A lentiviral expression vector pCDH-CMV-MCS-EF1a-GFP, CD513B-1 (Addgene, Cambridge, MA, USA) was digested with EcoRI and BamHI, and the purified fragments of DNMT3A and DNMT3B were inserted into lentiviral expression vector. The sequence of each construct was confirmed by Sanger sequencing.

To generate the lentiviral particles, lentiviral-based expression vector, packaging vectors pMDLg/pRRE and pRSV-REV, and pH27G as envelope plasmid were transfected into 293FT cell line using jetPRIME® transfection reagent (polyplus, Illkirch-Graffenstaden, France). The lentiviruses were transduced into THP-1 cells in the presence of 8 μg/mL polybrane (TR-1003, Sigma-Aldrich) and then selected using 5 μg/mL puromycin (Enzo Life Sciences, Inc.).

Statistical analysis
Data analysis for cell viability and cell cycle analysis were performed using one-way ANOVA followed by post hoc Tukey multiple comparisons or Bonferroni tests when appropriate. Spearman correlation test was conducted to evaluate the correlation between continuous variables. To evaluate the correlation between mRNA levels and cell viability in patient samples, we used Spearman’s rank correlation coefficient using ΔCt DNMTs (Ct DNMTs–Ct average of β-actin and 18s). Statistical analyses were performed using Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). For all analyses, the p values were 2-tailed, and p<0.05 was considered as statistically significant.

Results
Volasertib effectively inhibits proliferation of HMA-resistant cell lines
To assess the growth inhibition effect of volasertib against HMA-resistant cell lines, we treated MOLM/AZA-1, MOLM/DEC-5, and MOLM-13 with increasing concentrations of volasertib and compared the antiproliferative effects of volasertib with those of azacitidine or decitabine. After 72 h of treatment, volasertib was highly potent against all three cell lines as a single agent (Fig. 1A). When determining dose-dependent curves and the half-maximal inhibitory concentration (IC50) value of each drug, volasertib showed significantly lower IC50 value compared to azacitidine or decitabine, especially in HMA-resistant cell lines (Fig. 1B).

Volasertib shows synergistic effects with hypomethylating agents or cytarabine in HMA-resistant cell lines
Seeing the potential of single-agent volasertib in HMA-
Using the IC₅₀ value after 72 h treatment of each agent (Table 1). Next, we treated MOLM-13, MOLM/AZA-1, and MOLM/DEC-5 HMA-resistant cell lines (Table 1).

To evaluate the antileukemic mechanism by which volasertib induces combination effects when treated with HMAs or cytarabine, one of widely used intensive chemotherapeutic agents in MDS/AML. We determined the IC₅₀ concentrations of volasertib, azacitidine, decitabine, and cytarabine monotherapy, respectively using the IC₅₀ value after 72 h treatment of each agent (Table 1). Next, we treated MOLM-13, MOLM/AZA-1, and MOLM/DEC-5 cell lines with IC₅₀, higher (×5), and lower (×1/5) concentrations of each drug - azacitidine, decitabine, and cytarabine - in combination with volasertib simultaneously or as a single agent volasertib for 48 h (Supplementary Table 3).

Combined treatment of volasertib and cytarabine was synergistic in both HMA-resistant cell lines (MOLM/AZA-1 and MOLM/DEC-5), and volasertib in combination with azacitidine or decitabine showed significant synergism in MOLM/DEC-5 cell lines (Table 1).

**Volasertib induces caspase-dependent apoptosis in HMA-resistant cell lines**

To evaluate the antileukemic mechanism by which volasertib promotes cell death, we examined the status and degree of apoptosis in HMA-resistant cells. Volasertib treatment with a concentration of 100 nM dramatically increased the annexin V-positive cells proportion in both early and late apoptosis (Fig. 2A, 2B). As the expression of phospho-PLK (p-PLK) (T210) and phospho-histone 3 (p-H3) (S10) is known to be induced by volasertib treatment (Rudolph et al., 2015; Adachi et al., 2017), we assessed the protein expression of p-PLK and p-H3 in addition to apoptosis-related proteins. After treatment of 100 nM volasertib for 24 h, expression of p-PLK and p-H3 as well as cleaved caspase 8, 9, and 3, and PARP was significantly enhanced, whereas that of X-linked inhibitor of apoptosis (XIAP) decreased (Fig. 2C). The expression of apoptosis-inducing factor (AIF) was not significantly changed after volasertib treatment, suggesting that volasertib induces apoptosis in a caspase-dependent pathway.

**Volasertib facilitates apoptotic cell death independent of the G2/M cell cycle arrest**

The cell cycle accumulation in the G2/M phase followed by the sub-G1 phase was previously reported as a mode of action of volasertib (Rudolph et al., 2009; Adachi et al., 2017). To identify the mechanism of volasertib-induced apoptosis in HMA-resistant cell lines, we first assessed a cell cycle distribution by flow cytometry and cell cycle-associated protein expression using immunoblotting assay after 24 h of treatment with 10 and 100 nM of volasertib. As shown in Fig. 3A and 3B, we observed no significant cell cycle arrest in the G2/M phase after volasertib treatment compared to control, whereas the majority of cells accumulated in the sub-G1 phase and decreased in the G1 phase (p<0.0001). We also examined the expression of protein and mRNA transcripts associated with the G2/M cell cycle arrest. In line with the cell cycle assay results, there was no increase in the expression of p-ATM and p53 which are known to induce the G2/M arrest (Fig. 3C), and also no change was observed in mRNA expression (Supplementary Fig. 1). These results indicate that the main mechanism of volasertib in HMA-resistant cell lines inducing apoptosis is other than the G2/M cell cycle arrest.

**Volasertib decreases expression of PI3K/AKT/mTOR and ERK, and DNMT family**

To further explore the mechanism of action of volasertib on HMA-resistant cell lines, we investigated changes in related molecules in the phosphatidylinositol-3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MEK)/extracellular signal-related kinase (ERK) 1/2 signaling pathway. As shown in Fig. 4A, we found that endogenous phosphorylated PI3K/AKT/mTOR and MEK/ERK proteins were overexpressed in HMA-resistant cell lines compared to parental cell lines, and phosphorylation of the proteins involved in PI3K/AKT/mTOR and MEK/ERK pathways significantly decreased after volasertib treatment. As the DNMT family enzymes are upregulated in our HMA-resistant cell lines, we examined the effect of volasertib on DNMTs by immunoblotting assays. The expression of DNMT1, DNMT3A, and DNMT3B was dramatically suppressed by 24 h treatment of 100 nM volasertib in HMA-resistant cell lines (Fig. 4B). These results suggest that volasertib exerts its antiproliferative effects in HMA-resistant cell lines through inhibition of DNMTs and the signaling pathways of PI3K/AKT/mTOR and MEK/ERK.

**DNMT3B expression inversely correlates with the antiproliferative effect of volasertib in primary cells from patients with MDS or MDS/AML**

To evaluate the efficacy of volasertib against primary MDS or MDS/AML cells, we treated BM mononuclear cells collected from MDS (BM blast ≥5%) or MDS/AML patients in vitro with volasertib (n=42), decitabine (n=27), azacitidine (n=44), or cytarabine (n=24). Supplementary Table 2 shows the characteristics of 42 patients for BM samples. After 48 h exposure to 10 μM of each drug, volasertib showed comparable antiproliferative effects with decitabine, azacitidine, or cytarabine.
Volasertib induces apoptosis in MOLM-13 and HMA-resistant cell lines. The apoptotic effects in MOLM-13 and HMA-resistant cell lines following treatment with 10 nM and 100 nM volasertib for 24 h. The level of apoptosis was measured by using Annexin V-FITC/propidium iodide, and represented the distribution (A) and early and late apoptotic portion (B). Error bars represent the mean±SD of at least three independent experiments. ***p<0.01, *p<0.05 compared with control. (C) Immunoblot analysis of the expression levels of the volasertib-associated proteins (p-PLK and p-H3) and the apoptosis-associated proteins (AIF, XIAP, cleaved caspase 8, 9, and 3, and cleaved PARP) in all cell lines.

(Fig. 5A).

Next, we examined the mRNA expression of several genes involved in cell cycle regulation or epigenetic modification and analyzed the correlation of the expression with the antiproliferative effect of volasertib in primary MDS or MDS/AML cells. Interestingly, a higher expression of DNMT3b significantly correlated (p=0.006) with resistance to volasertib, but the expression of DNMT1, DNMT3A, and cell cycle-associated genes -p21, p27, and aurora kinase gene family (AKA/AKB/ACK)- did not (Fig. 5B, 5C). This result represent DNMT3B expression might affect the efficacy of volasertib.

**Overexpressed DNMT3B is associated with primary resistance to volasertib**

In order to evaluate how overexpressed DNMT3B affects the efficacy of volasertib, we established the DNMT3B-over-
expressed leukemic cell line using THP-1 cell lines (Fig. 6A, 6B). The mRNA and protein expressions increased in the lentiviral-DNMT3A (pDNMT3A) and -DNMT3B (pDNMT3B) transduced THP-1 cell lines, whereas there was no difference in expression levels in vector alone (pCMV) cell lines compared to parental THP-1 cell lines. As shown in Fig. 6C and 6D, pDNMT3B cell lines was more resistant to volasertib than pCMV cell lines showing 83-fold increased IC50 value compared to that of pCMV cell lines. We next evaluated the changes in PI3K/AKT/mTOR and ERK signaling pathway with volasertib treatment in the DNMT3B-overexpressed cell lines. In pDNMT3B cells, volasertib did not induce PARP or caspase cleavage, and there was no change in PI3K/AKT/mTOR and ERK expression after volasertib treatment (Fig. 6E). These findings indicate that overexpression of DNMT3B is closely related to resistance to volasertib.

**DISCUSSION**

In our HMA-resistant cell lines (MOLM/AZA-1 and MOLM/DEC-5), which have cross-resistance to both azacitidine and
decitabine, volasertib exerted potent antiproliferative activities in vitro. Volasertib also exhibited synergistic effects with cytarabine or hypomethylating agents in HMA-resistant cell lines. Combined treatment of volasertib and cytarabine also showed synergism in both MOLM/AZA-1 and MOLM/DEC-5 cell lines. In a previous study, volasertib in combination with cytarabine improved efficacy compared with either single agent in an AML patient-derived subcutaneous xenograft model (Rudolph et al., 2015). The phase II clinical trials of low-dose cytarabine with or without volasertib in AML demonstrated higher response rates with a combination of the two agents over low-dose cytarabine alone (Döhner et al., 2014). In other studies, the addition of azacitidine to volasertib was effective in most primary AML cells (Adachi et al., 2017), and the combination treatment obtained synergistic results in an AML cell line (MV4-11) and a xenograft model (Rudolph et al., 2015). Our findings suggest that volasertib alone or in combination with cytarabine or HMAs may provide evidences for overcoming inherent or acquired HMA-resistances in MDS or AML patients.

In previous studies, volasertib inhibits PLK1, PLK2, and PLK3 with IC\textsubscript{50} value of 0.87, 5, and 56 nM/L, and induces antiproliferative effects against colon cancer (HCT 116), lung cancer (NCI-H460), melanoma (BRO), and hematologic cancer (GRANTA-519, HL-60, THP-1, Raji, and MV-4-11) cell lines in vitro (Rudolph et al., 2009, 2015). Mode of action of volasertib is known to be related to inhibition of cell division, leading to apoptotic cell death (Golsteyn et al., 1994; Hamanaka et al., 1995). Regarding cell division, PLK1 immunofluorescence experiments revealed the prevention of proper localization of PLK1 to kinetochores and chromosomes by volasertib (Münch et al., 2015). Cancer cells treated with volasertib showed an accumulation of mitotic cells with formation of aberrant mono-

![Figure 4](image_url)

**Fig. 4.** Volasertib decreases expression of PI3K/AKT/mTOR and MEK/ERK pathway and DNMT family proteins. MOLM-13, MOLM/AZA-1, and MOLM/DEC-5 cells were treated with 10 nM and 100 nM volasertib for 24 h. Immunoblotting analysis of the PI3K/AKT/mTOR and MEK/ERK signal pathway proteins (A) and the DNMT family proteins (B) in all cell lines.
polar spindles and increased levels of p-H3, a mitotic marker that is phosphorylated during mitosis, indicating that cells were arrested in early M phase (Rudolph et al., 2009; Spartà et al., 2014; Gjertsen and Schöffski, 2015; Van den Bosch et al., 2016). Consistent with the findings, volasertib treatment changed the cell cycle distribution, as seen by an increased G2/M phase in the flow cytometry profiles, and the prolonged mitotic arrest eventually resulted in apoptosis (Rudolph et al., 2009; Spartà et al., 2014). In addition, antecedent studies revealed that expression of several genes are related to G2/M cell cycle regulation including p21, a p53-induced gene (Chan et al., 2000), GADD45 (Jin et al., 2002), CDK1 (Vassilev, 2006), and ATR/ATM (Beishline and Azizkhan-Clifford, 2014). However, volasertib-induced apoptotic cell death showed no causal relationship with expression changes of the G2/M checkpoint-regulating gene or protein and with the G2/M excluding sub-G1 cell cycle arrest in our HMA-resistant cell lines. The differences might come from the acquired cellular characteristics such as overexpressed DNMT3A or DNMT3B with regards to the drug resistance to HMAs, and further functional genomic studies are warranted.

In our HMA-resistant cell lines, phosphorylation of the DNMT family enzymes, PI3K/AKT pathway, and ERK increases, and the antiproliferative effects of volasertib were accompanied by the decrease of expression of these proteins. The PI3K/AKT pathway is known to regulate DNMT1 by phosphorylation of Ser143, which is mediated by AKT (Estève et al., 2011); AKT does not directly increase the methyltransferase activity of DNMT1, but paradoxically increases DNMT1 stability. The association of PI3K/AKT pathway and PLK1 has been studied in various situations. Some studies showed that overexpression of PLK1 induced activation of the PI3K/AKT pathway (Li et al., 2014; Cai et al., 2016), while others suggested that the PI3K/AKT pathway might be upstream of PLK1 (Mao et al., 2016; Wu et al., 2019). Although the relationship between PI3K/AKT pathway and PLK1 should be more clearly elucidated in future studies, the antiproliferative effects of volasertib in our HMA-resistant cell lines appear to be related to the interaction of PLK1, PI3K/AKT pathway, and DNMT enzymes. Besides, volasertib decreased p-ERK in our HMA-resistant cell lines. A study showed that PLK1 activated the ERK pathway in the epithelial-to-mesenchymal transition model (Wu et al., 2016).

DNMT family is a group of enzymes responsible for the attachment of a methyl group to the C-5-position of cytosine, and there are three main DNMTs: DNMT1, DNMT3A, and DNMT3B. Overexpression and promoter methylation of DNMT3B have been observed in several human cancer cells, suggesting an association between DNMT3B and tumor development. A study reported that gene amplification and protein overexpression of DNMT3B were associated with decreased sensitivity to HMAs, including decitabine and azacitidine in cancers of various situations. Some studies showed that overexpression of PLK1 induced activation of the PI3K/AKT pathway (Li et al., 2014; Cai et al., 2016), while others suggested that the PI3K/AKT pathway might be upstream of PLK1 (Mao et al., 2016; Wu et al., 2019). Although the relationship between PI3K/AKT pathway and PLK1 should be more clearly elucidated in future studies, the antiproliferative effects of volasertib in our HMA-resistant cell lines appear to be related to the interaction of PLK1, PI3K/AKT pathway, and DNMT enzymes. Besides, volasertib decreased p-ERK in our HMA-resistant cell lines. A study showed that PLK1 activated the ERK pathway in the epithelial-to-mesenchymal transition model (Wu et al., 2016).

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Future study needs to evaluate the possible role of DNMT3B as a biomarker for volasertib treatment.

In summary, volasertib showed significant antitumor activities through inhibition of DNMT3B and PI3K/AKT/mTOR and ERK signaling pathways in HMA-resistant cell lines as well as primary cells from patients with MDS or MDS/AML. Synergism of volasertib with cytarabine or HMAs was observed. Our results provide new insight into overcoming the HMA-resistance in MDS or MDS/AML patients.

**CONFLICT OF INTEREST**

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